Polymorphism of Glutathione S-transferase T1, M1 and P1 Genes in a Shanghai Population: Patients With Occupational or Non-occupational Bladder Cancer

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Objective Glutathione S-transferases are involved in the conjugation of xenobiotics. To explore whether GSTs polymorphisms are involved in the development of occupational or non-occupational bladder cancer, polymorphism frequencies of GSTT1, M1 and P1 were investigated in a normal population, which had been settled in a rural area in Shanghai suburb for at least 5 generations as well as in a group of patients with benzidine exposure related occupational bladder cancer in Shanghai dyestuff industry and a group of patients with non-occupational bladder cancer. **Methods** PCR based procedures were performed in the study populations to confirm the genotypes of GSTT1, M1 and P1. **Results** The polymorphisms at locus of GSTP1- A1578G in the normal population differed significantly from those in Caucasians or African Americans. All the subjects genotyped so far (n =118) bore only homogenous wild genotype (C2293/ C2293) at GSTP1 - C2293T locus. This locus seemed to be a monomorphic in Shanghai population. No significant difference in GSTT1 and GSTM1 polymorphic form frequencies could be confirmed among three groups of subjects. An overrepresentation of GSTP1 AG or GG genotype corresponding a less stable and less effective isozyme protein was detected in patients with benzidine related occupational bladder cancer, compared with that in the normal population though a statistical significance was not yet reached (P=0.09, OR=1.96, 95% CI 0.89-4.32,). **Conclusion** This study suggests that GSTM1 or GSTT1 homozygous deficiency genotypes and their combination do not have a clear impact on bladder cancer incidence in a Shanghai population. It seems that GSTP1 polymorphism is not associated with non-occupational bladder cancer. GSTP1 AG or GG genotype has a higher frequency in the patients with benzidine related occupational bladder cancer, and further work is needed to confirm if GSTP1 AG or GG genotype plays a role in the development of occupational bladder cancer.

Key word: Polymorphism; Glutathione S-transferase; Bladder cancer

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) catalyze the conjugation of endogenous reduced glutathione (GSH) with electrophilic center in the molecules of xenobiotics or their

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metabolites. As a result of conjugation, the hydrophilicity of the molecules increases and in most case, facilitates the excretion. Among the members of GSTs super-gene family[1] GSTT1, M1, P1 and M4 were proved so far to be polymorphic among the individuals of different races and different resident groups in the world [2, 3].

An overrepresentation of GSTM1 0/0 genotype in several patient groups with bladder cancer[4, 5, 6] suggested its possible association with higher susceptibility towards bladder cancer. So far, however, most of the studies were carried out in Caucasian subjects. To explore the possible impact of GSTs polymorphic genotype on the bladder cancer risk with or without connection to aromatic amines exposure in East Asia population, GSTT1, M1 and P1 gene genotyping was performed in two groups of patients diagnosed as bladder cancers: (1) Employees (or retired workers) of Shanghai dyestuff industry who were occupationally exposed to benzidine in workplaces at certain dose level before benzidine had been finally banned for any industrial purpose in 1976 [7]; (2) A group of bladder cancer patients who had never a career experience related with the exposure to benzidine or other aromatic amines.

MATERIALS

Subjects

All the subjects in this study were of ethnic Han origin (Chinese majority that represents 93% of the nation' s population).

Normal Population in Suburb of Shanghai (n=182*)*

Blood samples were collected in a rural area in Shanghai suburb. Donors' families had settled in this area for, at least, five generations. More than two donors from the same family were rejected. For a married woman, it was confirmed for the first time that her parents' home was within the same county or in the adjoining county (within 50-km distance). All the subjects had never been diagnosed to suffer from any kind of cancers, cardiovascular diseases, mental disorders or any other serious health problems at the time of sampling.

Subjects Without Bladder Cancer From an Benzidine Exposed Cohort in Shanghai Dyestuff Industry (n=317*)*

Benzidine, as an evident human bladder carcinogen, is listed in Group I of chemical carcinogens by the International Agency of Research on Cancer[8]. Synthesis of benzidine was first introduced in Shanghai in 1946 as a part of bazidine-based dye production process and had been widely used in this city for 30 years before it was finally forbidden for all industrial purposes in China in 1976. A research cohort with a total number of 700 former benzidine exposed workers in Shanghai dyestuff industry was established in 1984. A follow up study and regular surveillance had been persisting since then. According to an epidemiological investigation reported earlier on the same cohort, the standardized incidence ratio (SIR) of bladder cancer reached the level of 3 500 for the entire cohort, and even higher levels (up to 7 500) for the subgroup at the most risk working positions[7]. In 1999 only less than 400 persons were still under regular surveillance and 215 death were registered. The majority (79/215) died of various kinds of cancers, with bladder cancer as the first death cause (16/79). By the end of 1998, 317 individuals (age range: 61±10) in the cohort had not been diagnosed as bladder cancer patients although exfoliated urothelial cells from some subjects (69/317) revealed different pre-malignant cytological changes with a classifying

procedure according to Papanicolaou *et al*.[9].

Bladder Cancer Survivors Occupationally Exposed to Benzidine in Dyestuff Industry (n=29*)*

Twenty nine bladder cancer survivors from benzidine occupationally exposed cohort were included in this study.

Non-occupational Bladder Cancer Patient Group (n=32*)*

The subjects in this group were inpatients in the urological department of a local hospital at the time of sampling. Any possible occupational exposure history to benzidine or other aromatic amines was excluded by questionnaire.

METHODS

Sample Collection and DNA Extraction

Blood sampling and DNA extracting were performed based on the procedure described previously[10]. EDTA was used as blood anticoagulant. Genomic DNA from leukocytes was prepared by dialysis of erythrocytes, incubation with proteinase K, chloroform extraction, ethanol precipitation, subsequently.

GSTT1 and M1 Genotyping

The genotyping of GSTT1 and GSTM1 in one tube procedure was taken[11]. GSTM1 gene fragment of 215bp was amplified with a set of primers: 5'- GAA CTC CCT GAA AAG CTA AAG C -3' and 5'- GTT GGG CTC AAA TAT ACG GTG G -3' together with 480bp fragment of 3' part of GSTT1 gene (with a pair of primers: F1143: 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and F1144: 5'-TCA CCG GAT CAT GGC CAG CA-3'A) and a 268bp fragment of human β-globin gene serves as internal control (primers: PC04: 5'-CAA CTT CAT CCA CGT TCA CC-3' and GH20: 5'-GAA GAG CCA AGG ACA GGT AC-3'). Reaction was conducted as initial melting at 94℃ for 5 min followed by 35 cycles consisting of melting at 94℃ for 30s, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min. A final extension was lasted for 10 min at 72℃ to end the procedure.

GSTP1 Genotyping

GSTP1-$A_{1578}G$ ($I_{105}V$) locus: The genotyping procedure was similar to that of Harris *et al*., 1998[12]. PCR amplification was conducted with a pair of primers: 5′-CGC ATG CTG CTG GCA GAT CAG-3′ and 5′ -CAA GCC ACC TGA GGG GTA AGG-3′ at the temperature program as following: initial melting at 94℃ for 5 min, 35 cycles of melting at 94℃ for 1 min, annealing at 64℃ for 1 min, extension at 72℃ for 2 min, followed by a final extension at 72℃ for 10 min. Amplification product (851bp) was then digested with *BsmA I* (from New England Biolabs) at 55℃ for 2 h. After that 10 mmol/L EDTA was added to terminate the reaction.

GSTP1-$C_{2293}T$ ($A_{114}V$) locus: The genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Harris *et al*.[12]. A pair of primers were used: 5′-GTT GTG GGG AGC AAG CAG AGG –3′for forward

and 5′-CAC AAT GAA GGT CTT GCC TCC C –3′ for reverse. The PCR was carried out with initial melting at 94℃ for 5 min, then 35 cycles of denaturing for 30 seconds at 94℃, annealing for 30 seconds at 60℃, and extension 1 min at 72℃, followed by a final extension at 72℃ for 10 min. A product of 217bp was then digested with restriction endonuclease *Cac8* I (from New England Biolabs) at 37℃ for 2 hours, using 10mmol/L EDTA to terminate the reaction.

All the primers used were synthesized by Gibco-BRL Life Technologies (Grand Island, NY, USA).

Electrophoresis

The products of PCR amplification and restriction digestion were separated and identified for GSTT1, M1 and GSTP1 genotypes by electrophoresis on 2% agarose gel or 3.2% agarose 3:1 HRB (from AMRESCO), respectively.

RESULTS

The GSTT1, GSTM1 and GSTP1 Polymorphism in the Normal Population in Shanghai Suburb

In the normal population, the frequencies of GSTT1 0/0 and GSTM1 0/0 genotype were 48.5% and 54.4%, respectively. While the frequency for GSTM1 homozygous deficiency (54%) was within the range reported for most of other racial groups (~50%) in the world, and the GSTT1 0/0 genotype (48%) was over presented in this group than in Caucasian population (10%-20%). No statistic ally significant difference in GSTT1 0/0 (P=0.85) and GSTM1 0/0 (P=0.27) genotypes was found between this group and a group of residents in Shanghai urban (49.3%, and 48.9%, respectively[10]) (Table 1).

TABLE 1

GSTT1and M1 Homozygous Deletion Genotype in the Normal Population in Shanghai Suburb

Gender	Genotype		
	GSTT1 0/0	GSTM1 0/0	GSTT1 0/0- GSTM1 0/0
Male	43.8% (39/89)	49.4% (44/89)	24.7% (22/89)
Female	52.7% (49/93)	59.1% (55/93)	34.4% (32/93)
Total	48.4% (88/182)	54.4% (99/182)	29.7% (54/182)

In this population, the mutation allele frequency at loci $A_{1578}G$ and $C_{2293}T$ of GSTP1 gene, seemed to be lower than that in Caucasians and African Americans. G_{1578} allele (corresponding to low catalyzing activity phenotype V_{105}) only represented 22% of the population frequency. For locus $C_{2293}T$, the mutation allele could be detected neither in homozygous nor in heterozygous form in all the subjects tested (n =118) (Table 2).

TABLE 2

GSTP1 Gene Polymorphism in the Normal Population in Shanghai Suburb[a]

Groups	I105/I105 (AA)	I105/V105 (AG)	V105/ V105(GG)	*n*	*P*-value	A114/ A114 (CC)	A114/ V114 (CT)	V114/ V114 (TT)	*n*	*P*-value[b]	Literature
Shanghai	110 (61.4%)	59 (33.0%)	10 (5.6%)	179	Ref.	118 (100%)	0 (0%)	0 (0%)	118	Ref.	This work
Beijing	32 (65.3%)	15 (30.6%)	2 (4.1%)	49	0.85	49 (98.0%)	1 (2.0%)	0 (0%)	50	0.28	Harris MJ *et al.*1998[12]
Taiwan	78 (67.2%)	35 (30.2%)	3 (2.6%)	116	0.37	–	–	–	–	–	Watson MA *et al.*1998[13]
African American	48 (35%)	63 (46%)	26 (19%)	137	1.8×10^{-6}	106 (94.6%)	6 (5.4%)	0	112	0.07	Watson MA *et al.*1998[13]
European American	119 (42%)	147 (52%)	21 (7%)	287	1.0×10^{-4}	93 (81.6%)	20 (17.5%)	0	114[c]	0.001	Watson MA *et al.*1998[13]
European Australian	80 (40.2%)	101 (50.8%)	18 (9.0%)	199	2.0×10^{-4}	170 (85.4%)	29 (14.6%)	0	199	0.003	Harris MJ *et al.*1998[12]

Note. [a]*chi*-square test was used, [b]Comparison only for alleles, [c]sic.

GSTT1, GSTM1 Polymorphism Among Members Without Bladder Cancer in Benzidine Occupationally Exposed Cohort

The frequencies of GSTT1 0/0, GSTM1 0/0 and their combination among the members without bladder cancer in the benzidine occupationally exposed cohort were 55.5%, 56.8% and 33.8%, respectively. The result showed no significant difference in the genotypes of GSTT1 0/0 (P=0.16), GSTM1 0/0 (P=0.61) or the combination (P=0.35) between a group of subjects without bladder cancer in a benzidine occupational exposed cohort compared with controls (Table 3).

TABLE 3

Distributions of Genotypes of GSTT1 and GSTM1 Among Non-diseased Members in Benzidine Occupationally Exposed Cohort[a]

Genotype	Exposed Cohort (n=317)	Control Population (n=182)	OR[b] (95% CI)	*P*-value
T1 0/0	55.5% (176/317)	48.4% (88/182)	0.78 (0.55-1.11)	0.16
M1 0/0	56.8% (180/317)	54.4% (99/182)	1.10 (0.76-1.59)	0.61
T1 0/0-M1 0/0	33.8% (107/317)	29.7% (54/182)	1.21 (0.82-1.79)	0.35

Note. [a]*chi*-square test was used, [b]Odds ratio, and 95% confidence interval.

GSTT1, GSTM1 and GSTP1 Polymorphism in A Group of Benzidine Exposure Related Occupational Bladder Cancer Patients and A Group of Non-occupational Bladder Cancer Patients

The distribution of GSTT1 0/0, GSTM1 0/0 and GSTT1 0/0-GSTM1 0/0 genotype was similar in these two bladder cancer groups and the control. Figures were displayed in Table 4.

TABLE 4

GSTT1 and M1 Polymorphisms Among Normal Population, Benzidine Exposure Related Occupational Bladder Cancer Patients and Non-occupational Bladder Cancer Patients in Shanghai[a]

Populations	Genotype			
	GSTT1 0/0	GSTM1 0/0	GSTT1 0/0-GSTM1 0/0	*P*-value
Non-occupational Bladder Cancer Patients	46.9% (15/32)	62.5% (20/32)	25.0% (8/32)	0.77
Occupational Bladder Cancer Patients	48.3% (14/29)	58.6% (17/29)	27.6% (8/29)	0.95
Normal Population	48.4% (88/182)	54.4% (99/182)	29. 7% (54/182)	Ref.

Note. [a]*chi*-square test was used.

For the GSTP1 $A_{1578}G$ locus, the non-occupational bladder cancer patients had similar AG or GG genotype frequency with the control (because the genotype frequency of GG was very limited, the AG and GG genotype were combined to calculate *P*-value and OR). However, the occupational bladder cancer patients had elevated AG or GG genotype frequency as is shown in Table 5.

TABLE 5

Comparison of Occupational and Non-occupational Bladder Cancer Patients and A Normal Population at Locus $A_{1578}G$ of GSTP1 Gene[a]

Populations	Genotypes of GSTP1 (Locus $A_{1578}G$)				OR[b] (95% CI)	*P*-value
	AA	AG	GG	AG + GG		
Normal Population	110 (61.4%)	59 (33.0%)	10 (5.6%)	69 (38.5%)	Ref.[c]	Ref.
Non-occupational Bladder Cancer Patient	20 (62.5%)	11 (34.4%)	1 (3.1%)	12 (37.5%)	0.96 (0.44-2.09)	0.91
Occupational Bladder Cancer Patient	13 (44.8%)	16 (55.2%)	0 (0%)	16 (55.2%)	1.96 (0.89-4.32)	0.09

Note. [a]*chi*-square test was used, [b]Odds ratios were calculated by comparison of the normal population and cancer groups for GSTP1 A/G or G/G versus GSTP1 A/A. [c]The distribution of genotypes among normal population was in Hardy-Weinberg equilibrium.

DISCUSSION

Statistically significant difference in GSTT1 and GSTM1 could not be confirmed between this investigated suburb normal population and a group of Shanghai urban residents. The latter took shape more recently. The worry about the un-homogenization of genetic background in modern population of "immigrants' city" Shanghai could be somewhat neglected.

No mutation allele of GSTP1 $C_{2293}T$ was found in all subjects tested, suggesting that this locus might be monomorphic in the population. The AG or GG genotype frequency

profile for $A_{1578}G$ locus in this population significantly differed from that of Caucasians ($P<0.001$) and African Americans ($P=1.8\times10^{-6}$). It was quite similar to the resident groups in Beijing ($P=0.85$) and Taiwan ($P=0.37$), suggesting that the polymorphic profile of human GSTP1 gene among different Chinese subgroups is highly homogeneous.

An overrepresentation of GSTM1 0/0 genotype in several bladder cancer patient groups[4-6] suggests a possible association with higher susceptibility of bladder cancer. So far most of the authors have conducted their studies only in general population. Studies were carried out in diagnosed bladder cancer patient groups and healthy general subjects as the controls. Rothman *et al*. [14] reported a case-control study on bladder cancer among workers previously exposed to benzidine in several Chinese cities, including Shanghai. No overall increase in bladder cancer risk related with GSTM1 0/0 genotype was found among 38 bladder cancer cases and 43 controls (OR=1.0, 95% CI 0.4-2.7). A complementary study showed that GSTM1 enzyme protein was not bound to benzidine or its metabolites *in vitro*. This study failed to find significant difference in the distribution of GSTT1 0/0, GSTM10/0 or their combination between the members without bladder cancer in the benzidine-exposed workers and the normal population. It should be noticed that all the subjects listed in Table 3 were still in healthy or sub-healthy state and could be considered as the survivors of cohort's long-time "natural depletion". They might represent a less susceptible portion to benzidine exposure in the whole original cohort. The present data suggest that GSTM1 or GSTT1 homozygous deficiency genotypes and their combination have no significant impact on bladder cancer incidence in a former benzidine exposure cohort in Shanghai, providing a contrast to the assumed association with elevated bladder cancer risk in the general Caucasian population.

From the GSTT1 and GSTM1 gene polymorphism genotyping in the two groups of bladder cancer patients, no significant difference could be confirmed in the major polymorphic forms, though the slightly more GSTM1 homozygous deficiency might be observed in patients with non-occupational bladder cancer. Due to the limited cases, more work is required in the future.

A higher frequency of AG or GG genotype was displayed in patients with benzidine related occupational bladder cancer compared with the normal population, though no statistical significance was found ($P=0.09$, OR=1.96, 95% CI 0.89-4.32). The gene with a base transition A_{1578} G codes a mutant enzyme protein with an amino acid substitution of I_{105} **V in substrate binding center, which represents a less stable and less effective** isozyme form in the organisms.

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